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The purpose of this project is to optimize and apply new methodology for the detection of unknown DNA adducts in breast and, for comparison, other human tissues. Current methodology for this purpose has shortcomings, and breast cancer may be initiated by unknown DNA adducts. Initially the methodology will be applied to nonpolar adducts, and later it can be expanded. The concept for the new methodology is the same as ^{32}P-postlabeling, but chemical labeling is done with a fluorescent tag to overcome variation in labeling yields, increase resolution, and provide compatibility with mass spectrometry. As part of optimization, a prototype fluorescent tag has been characterized in terms of its structural and reactivity properties. This has led to the detection of 60 fmol of dAMP by the method. Also, to facilitate purification of the tag, and improve removal of excess tag after the labeling reaction, its separation has been studied by immobilized metal affinity chromatography. This is intended to bring the sensitivity of the method closer to the low amol level that we have reached for standards.

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Roger Green 7/2/97

PI - Signature Date

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INTRODUCTION

DNA adducts, the consequence of covalent damage to DNA as by toxic chemical or physical conditions, play an important role in carcinogenesis and mutagenesis (1, 2). This is because DNA is an ultimate target in the body for agents causing these events. Thus it is important to measure DNA adducts comprehensively and accurately in human tissue as a way to learn more about the origins of disease processes having a genetic component such as human breast cancer.

DNA adducts are measured currently both as known and unknown compounds. The methodology available for measuring known DNA adducts, while not perfect, is adequate in many cases. However, current methodology for detecting unknown DNA adducts has severe shortcomings. Basically what is available for this purpose is ^{32}P-postlabeling, involving separation of the adducts, once they are ^{32}P-postlabeled, by thin layer chromatography (TLC) or high performance liquid chromatography (HPLC). The problems with ^{32}P-postlabeling for detecting unknown DNA adducts are as follows. First, unknown adducts are labeled to different, unknown degrees, so there is no guarantee that all the adducts are detected, nor even whether the more prominent spots or peaks represent the prominent adducts. Second, for unknowns, the method has been applied only to bulky adducts, since these are the easiest to detect. Third, because of the radioactivity, the separation techniques employed are kept simple, which in turn limits the resolution and characterization. Fourth, the limited resolution can make it difficult to compare results from different laboratories for unknown adducts. Finally, the method does not identify unknowns except by comparison with known adducts.

Sorting out the origins of breast cancer may require the detection and characterization of unknown DNA adducts. Thus we have set out in this project to develop improved methodology for detecting unknown DNA adducts in breast tissue. As a starting point, taking into account the information cited below, and to provide a comparison with data from ^{32}P-postlabeling, we will first apply the method to bulky DNA adducts in breast tissue. Subsequently the methodology can be expanded to give comprehensive detection of unknown DNA adducts.

The concept of the new methodology is the same as that of ^{32}P-postlabeling, but the details are quite different. A fluorescent tag is used in place of ^{32}P, chemical rather than enzymatic labeling is done, and capillary electrophoresis is used as a supplement to an HPLC separation to achieve high resolution. Final detection results from laser-induced fluorescence rather than radioactivity.

In principle, this overcomes or mitigates all of the above limitations of ^{32}P-postlabeling. It helps indirectly to characterize the unknowns since, once an interesting adduct is found, it can be scaled up and put into a mass spectrometer as a fluorescently-labeled product. In most laboratories, including ours, one would never put a ^{32}P-postlabeled compound into a mass spectrometer. At an earlier stage in our project we demonstrated the detection by mass spectrometry of a deoxynucleotide labeled with our fluorescent dye.

Little is known about DNA adducts and breast cancer, although it is generally considered that they must play a major role in this disease (3-5). Malins and coworkers have studied oxidative damage to DNA in both normal and cancerous human breast tissue (6). They found that the latter tissue contains a higher concentration of the oxidative adducts 8-oxoadenine, 8-oxoguanine, and 5-hydroxymethyluracil. Also the ratio of the 8-oxoadenine product to the corresponding ring-opened purine (which can form reductively from the 8-oxoadenine product) was higher in cancerous *vs.* normal tissue. This latter observation is consistent with the production of elevated levels of hydrogen peroxide by tumor cells (7). The amounts of these oxidative adducts in normal breast tissue were reported to be about 1-5 adducts in 10^4 normal nucleotides. Other adducts in biological samples in general are encountered at lower levels, e.g., 1 in 10^7 - 10^8 nucleotides (1,2).

Some other, more general observations may be relevant as well to a relationship between DNA adducts and breast cancer: the increasing incidence of this disease (5); its promotion (in rodents) by steroid hormones (4); the occurrence of genetic events such as oncogene activation in breast cancer (8); the existence of certain risk factors such as exposure to ionizing radiation and alcohol use (5); the ability of environmental xenobiotics such as polyaromatic hydrocarbons to induce mammary cancer in rodents (9) coupled with the fact that such agents tend to accumulate in human breast fat (10); and the increased incidence of mammary tumors in rodents on a high fat diet coupled with a suggested correlation between a human diet rich in fat (and protein) and higher DNA content of breast cancer cells (11).

EXPERIMENTAL

Materials

BO-IMI (12), C8-[N-acetyl-N-(2-fluorenyl)]amino-5'-dGMP (13) (C8-AAF-5'-dGMP), C8-oxo-5'-dAMP (14), and C8-benzyloxy-5'-dAMP (14) were prepared as described. 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl hydrazide (BODIPY FL C_3 hydrazide) was from Molecular Probes (Eugene, OR). Sodium tetraborate (BORAX), tris[hydroxymethyl]-aminomethane (TRIS), 1-ethyl-3-(3'-N,N-dimethylaminopropyl)carbodiimide (EDC), 2-(N- morpholino)ethanesulfonic acid (MES), 5'-dAMP, 5'-dCMP, 5'-dGMP, and 5'-TMP were purchased from Sigma Chemical Co. (St. Louis, MO).

HPLC-grade acetonitrile, methanol and propyl sulfonic acid silica (40 μm, 60 Å) were from J. T. Baker Inc. (Phillipsburg, NJ). Ammonium acetate (NH_4Ac) was from Fluka (Buchs, Switzerland). 5'-pCAAAGCTTG (a DNA oligomer) was from Oligos Etc. Inc. (Wilsonville, OR). Microcentrifuge tubes (1.5 ml, 05-407-10, Fisher Scientific, Pittsburgh, PA) were used for the BO-IMI reactions.

Buffers

Buffer A (pH 6.0): 0.5 ml of 0.2 M MES, 0.38 ml of 0.1 M NaOH, and 9.1 ml of water.

Buffer B (pH 6.0): 2.0 ml of 0.2 ml of 0.2 M MES, 0.28 ml of 0.5 M TRIS, 4.0 ml methanol, and 34 ml of water.

Buffer C (pH 8.7): 0.8 ml of 0.5 M boric acid, 0.8 ml of 0.5 M TRIS, 4 ml acetonitrile, and 34.4 ml of water.

Buffer D (pH 10.4): 2.0 ml of 0.05 M BORAX, 1.8 ml of 0.1 M NaOH, 5 ml of acetonitrile, and 41.2 ml of water.

Equipment

A home-built capillary electrophoresis (CE) apparatus with laser-induced fluorescence detection (Ar ion laser with excitation at 488 nm) was used (15). The CE unit was interfaced to a Macintosh Centris 610 computer through DYNAMAX MacIntegrator I (Rainin Instrument Co., Inc., MA). One of the contact input ports was used to trigger the data acquisition, and one of the contact outputs was used to interlock and trigger the regulated high-voltage dc power supply (Glassman High Voltage Inc., NJ). CE was performed in a 70 cm long fused-silica capillary (75 μm I.D.) with the detection window 45 cm from the injection end. Samples were injected hydrodynamically: anode end 5 cm higher for 20 sec (about 10 nL).

Fluoride Measurement of Hydrolyzed BODIPY Hydrazide.

An Orion 501 digital ionalyzer and an Orion F^- ion-selective electrode (Orion Research Inc., Cambridge, MA) were used for F^- measurement. Fluoride standard solutions, which gave a linear calibration curve, were prepared by weighing KF (MCB, Norwood, OH), dissolving it to 0.1 M in 0.1 M potassium phosphate, pH 6.5, and making dilutions in this buffer to 10^{-5} M fluoride. After 1.0 mL of a 8.9×10^{-4} M solution of BODIPY hydrazide in 0.01 M NaOH was kept for 0.5 h at room temperature, it was diluted with 9 mL of the 0.1 M phosphate buffer and tested, revealing a fluoride concentration of 1.8×10^{-4} M (1.8×10^{-3} M in the original solution).

Cation Exchange Filtration of a BO-IMI/Nucleotide Reaction Mixture

BO-IMI (1.2×10^{-2} M), 5'-dAMP (1.2×10^{-1} M), and EDC (1.2×10^{-1} M) in buffer A, 10 μL each, were briefly mixed and then kept at room temperature in the dark for 15 min. The starting BO-IMI was removed by loading the reaction mixture onto a Pasteur pipet column containing ~140 mg propyl sulfonic acid silica retained on glass wool, and eluting (with air pressure from

a rubber bulb, including a terminating flow of air through the column) with 2 x 0.3 ml of buffer A. Before this separation was performed, bulk cation exchanger in this buffer was adjusted to pH 6 with 0.1 M NaOH, and, after the column was prepared, it was washed with 10 mL of buffer A.

Detection of 60 and 600 Fmol of 5'-dAMP

BO-IMI (1.2×10^{-2} M), 5'-dAMP, and EDC (9×10^{-2} M), 1 µL each in buffer A, were mixed, and the resulting solution was kept dark at room temperature for 2 h. After the reaction mixture was diluted with 0.5 mL of pH 7.0 0.01 M NH_4Ac (pH adjusted with NH_4OH), it was loaded onto a cartridge column (see below). A rubber bulb was employed to push all the liquid out of the cartridge into a collection vial. A second 0.5 mL NH_4Ac was applied and similarly pushed into the vial. The collected solution was evaporated to dryness in a Speed-Vac (Savant Instruments). Fifty µL of buffer D was added followed after 30 min by injection into the CE capillary. To prepare the cartridge column, bulk cation exchanger in 0.01 M NH_4Ac was adjusted to pH 7.0 with 0.1 M NaOH, and loaded (140 mg) into a Pasteur pipet plugged with glass wool followed by washing with 10 mL of pH 7.0 0.01 M NH_4Ac.

Separation of BO–IMI and BO–HZ by IMAC

Sepharose-IDA in ethanol (1.5 ml) was packed into a 10 ml disposable pipet plugged with glass wool. After the column was washed with 20 ml of distilled water, 10 ml of 0.2 M metal sulfate (copper, nickel or zinc) were applied. The unbound metal ions were washed off with 20 ml each of acetate and distilled water, and the column was equilibrated with 20 ml of phosphate. A 100 µl sample which contained 10 µg of BO-IMI and 10 µg of BO–HZ in phosphate was loaded onto the column and eluted with a stepwise pH gradient, starting from phosphate and followed by acetate/HCl buffers: pH 6, 5, 4, 3 and 2. The fractions were tested by capillary electrophoresis with laser-induced fluorescence detection (CE–LIF). The separation was achieved in a 65 cm x 75 µm I.D. capillary (40 cm to detector) at 25 kV with anodic siphoning injection (column elevated 5 cm for 20 sec). The running buffer is 0.01 M Tris/MES with 10% acetonitrile, pH 6.2.

Preparation of zinc-free solvents and tubes

Water and buffers for the following experiment involving zinc measurements were made metal-free by extraction with dithizone in chloroform. Residual chloroform was removed by N_2 bubbling. Nitric acid (15%) soaking followed by washing with metal-free water was used to make the test tubes metal-free.

BO-HZ on Zn(II)-IMAC followed by Zn measurement

Sepharose-IDA gel (1.5 ml) was packed into a 5 ml disposable pipet and washed with 20 ml water. After adding 2 ml of 0.2 M $ZnSO_4$, 20 ml of acetate

and 50 ml of phosphate were applied to wash out unbound zinc. BO-HZ (15 μg) in 0.5 ml of phosphate was loaded onto the column and eluted with 25 ml of phosphate. Fractions were collected and analyzed for zinc content by atomic absorption spectroscopy.

ASSUMPTIONS

The basic assumption in this project is that DNA adducts initiate breast cancer. Since current methodology for measuring unknown DNA adducts has limitations, it is important to develop improved methodology for this purpose.

We are further assuming, although only as a starting point, that nonpolar DNA adducts explain the epidemic of breast cancer. Therefore we will apply to the methodology first to this class of adducts.

PROCEDURES

Our basic scheme for detecting DNA adducts in human samples consists of three general steps. In step I, standard techniques are used to isolate the DNA from a biological samples, hydrolyze the DNA enzymatically to nucleotides, and separate the nucleotides by HPLC with UV detection. Peaks for the normal nucleotides are visible on the HPLC chromatogram while DNA adducts escape detection because UV detection is not very sensitive. In step II, the late elution fraction from the HPLC separation, containing the bulk of the nonpolar adducts, is collected, and the DNA adduct nucleotides are covalently labeled with a fluorophore. In step III, the sample is subjected to capillary electrophoresis (CE) with laser fluorescence detection. DNA adducts are revealed as fluorescent peaks.

We plan to compare the adduct spectra of breast *vs* other tissues, and look for consistent peaks in the CE electropherograms that distinguish the breast DNA. If such adducts are found, then we can test whether they correlate with risk factors for breast cancer. Finally, samples can be scaled up so that mass spectrometry can be employed to characterize interesting DNA adducts that seem to be tied to breast cancer.

RESULTS AND DISCUSSION

Previously we pointed out that both the N1 and N3 nitrogens of the imidazole moiety of BO-IMI (**1**; the structures of our compounds are presented in Table 1) react with a phosphomonoester (12). For 5'-dAMP and 3'-dAMP (and the other corresponding mononucleotides that were studied), the ratio of the major (**3**) to minor (**2**) product was 97:3 and 98:2, respectively. It was assumed (but not proved) that this product ratio arose kinetically as a consequence of steric effects, tentatively making **3**=N1 isomer and **2**=N3 isomer for BO-IMI-5'-dAMP (see Table 1 for a definition of the N1 and N3 positions). To obtain this data, we conducted the labeling reaction at pH 6.0 for three hours, and then subjected the product mixture to capillary electrophoresis at the same pH. This separated the pair of isomeric products,

apparently due to a difference in the pKa's of their imidazole moieties in this pH region. For example, the imidazole moiety of guanosine 5'-phosphoimidazolide has a pK_a of 6.07 at 37°C (16). At an elevated pH ($\geq$ 8.7 was examined) the isomeric products comigrated, conveniently allowing a single peak to be observed for each target phosphate compound that was tested.

When the reaction mixture (at pH 6.0) is directly examined by CE-LIF after a shorter time period, e.g. 0.3 hours, as shown in Figure 1A, a higher ratio of **2** relative to **3** is seen relative to what is observed after 3 hours (Figure 1B). Thus there is a shift with time from a kinetically to a more thermodynamically-controlled product mixture. Further observations, about to be described, suggest that the primary mechanism for this shift involves preferential hydrolysis at pH 6.0 of the less stable isomer (**2**), which releases the 5'-dAMP for a second round of coupling to BO-IMI (excess EDC, a water soluble carbodiimide, is present).

We subjected a reaction mixture (containing a 10-fold molar excess of 5'-dAMP over BO-IMI) after 15 minutes to cation exchange filtration, which removed the residual EDC (but apparently not any pre-formed EDC-5'-dAMP, since it lacks a net charge). The collected sample was divided into two parts (A and B). Part A was immediately treated with an amount of BO-IMI equivalent to what was present initially; this adjustment was done without changing the pH. Buffer A was added to part B to keep the volume the same. The samples were stored at room temperature in the dark, while aliquots of each were tested periodically by CE to monitor the ratio of isomeric products. Thus we were testing the effect of BO-IMI on the ratio of **2** to **3** in the absence of intact EDC, to avoid the complication of hydrolyzed **2** or **3** re-reacting with EDC. After 20 hours, the pH of the A and B parts were 6.23 and 6.40, respectively.

As the absolute peak area for **2** decreased progressively in B (the part not supplemented with BO-IMI) throughout this storage period (first order kinetics; $t_{1/2}$ = 2.3 h), the peak for BO-IMI increased correspondingly, while that for **3** decreased to a lesser degree. (Previously we determined that the half-life for **3** at pH 6.0 is 19.7 hours (12).) In part A (supplemented with BO-IMI), four-fold less **2**, and slightly more **3** (about 10%), was present at the first time point (1.1 hours after the filtration step) than in part B containing no added BO-IMI. Apparently the added BO-IMI in part A was doing two things: reacting with EDC-5'-dAMP (which accounts for the initial, slight increase of **3** in A) and also catalyzing the hydrolysis of **2** (which both lowers the pH and the yield of **2** in A relative to what happens in B). Thus, both spontaneous and BO-IMI-catalyzed hydrolysis of the less stable isomer **2** in the ordinary reaction mixture (where excess EDC is present) appear to drive its conversion, via reactivation with EDC, to **3**. In the ordinary reaction (which is usually conducted for 3 hours), this thereby leads to the 97:3 product ratio of **3:2**.

Other known catalysts for hydrolysis of a phosphoimidazolide of a nucleotide include both $H_2PO_4^-$ and HPO_4^{2-} (16).

Assuming, as before, that **2** is the N3 isomer, then why does this isomer hydrolyze more rapidly at pH 6.0? It is known that a phosphoimidazolide hydrolyzes more rapidly when the imidazole moiety is protonated (16). We speculate that the postulated N3 isomer hydrolyzes faster since protonation at its free N1 site can be stabilized by solvation with little interference from the remote C4 alkyl group, in contrast to the behavior of the N1 isomer.

The electropherogram shown in Figure 2A basically corresponds to those shown in Figure 1 except that a small amount of BODIPY FL C_3 hydrazide, **4**, is present as a neutral marker. When the pH of this reaction mixture is raised to 10.4 and then, one hour later, returned to pH 6.0, the electropherogram shown in Figure 2B is observed. Co-injecting the two reaction mixtures gives the electropherogram shown in Figure 2C. Considering the relative peak positions as well as areas, it certainly appears that compounds **2** and **3** are converted to **2'** and **3'**, respectively, by the intermediate exposure to high pH. (Shortly we will also discuss compounds **1'** and **4'**.)

What happens at an elevated pH is that the two fluorine atoms attached to the boron are replaced by hydroxy groups. This was demonstrated in two ways. First, a fluoride ion selective electrode was used to determine that 2.0 equivalents of fluoride are formed when hydrazide **4**, as a pure sample, is exposed to alkaline pH. Second, appropriate protonated molecule and fragment ions were observed for compound **4'** by fast atom bombardment mass spectrometry (data not shown). Compound **4** and its hydrolysis product **4'** co-migrate at both pH 6.0 and 10.4 by CE (data not shown), demonstrating the neutrality of the dihydroxy product **4'**. Also seen in Fig. 2 is a peak for **1'**, the dihydroxy version of BO-IMI.

The four compounds **2, 2', 3, 3'** give rise to "two peaks" (**2+3** as one peak and **2'+3'** as the other) by CE at high pH as shown in Figure 3A. The complicated peak pattern reflects the hydrolysis of **2+3** to **2'+3'** during the electrophoretic separation. The sample in this case was injected into the CE column without prior incubation at an elevated pH to first bring the conversion to completion. As seen in Figure 3B, even 0.5 hour of such incubation converts nearly all of compounds **2+3** to **2'+3'**. Other peaks are absent in the electropherogram shown in Figure 3 (unlike Figure 2) because the sample was filtered over the silica cation exchanger prior to the separations shown in Figure 3.

Some of the BO-IMI forms a conjugate with EDC during the course of the reaction. Combining BO-IMI and EDC in the absence of 5'-dAMP, and

subjecting the resulting sample, without cation exchange filtration, to CE separation at pH 10.4, gives the electropherogram shown in Figure 4A. Since the sample was incubated at pH 10.4 for 1 hour prior to the separation, the peaks shown correspond to dihydroxy versions of the boron chromophore. To confirm the identity of this side product, a sample of a reaction mixture was subjected to fast atom bombardment mass spectrometry, leading to the mass spectrum shown in Figure 5. No incubation at pH 10.4 was done for the latter sample, so the species detected was the difluoro compound. The peak at m/z 174 apparently arises from reaction of the released m/z 156 moiety, a carbodiimide, with water to form the corresponding urea, as indicated in the figure.

In Figure 4B, we see the consequence of incubating, in 0.1 M HCl for 5 hours, the above reaction mixture (Fig. 4A) of BO-IMI plus EDC, prior to analysis of this sample by CE at pH 10.4. This causes some hydrolysis of **5** to reform **1** (resulting in more **1'** relative to **5'** in Fig. 4B). This is consistent with the known hydrolytic instability of an imidazole-carbodiimide conjugate at low pH (17).

The arrow in Figure 4B shows the migration position of a neutral marker (BODIPY FL C_3 hydrazide). Thus both compounds **5'** and **1'** have a net negative charge at this pH. There is no known pKa for imidazole in this pH region. BODIPY FL C_3 hydrazide and its dihydroxy analog have essentially the same migration times at this pH, so the charge cannot arise from the dihydroxyboron moiety. Thus some ionization of the dicarbohydrazide component of **5'** and **1'** must be taking place. To support this hypothesis, we acetylated BODIPY FL C_3 hydrazide in acetonitrile with acetic anhydride, and observed that the product, **6'**, unlike dihydroxy BODIPY hydrazide, **4'**, migrates as a negatively charged species at pH 10.4 (see inset in Fig. 4B; **4'** is migrating as a neutral in the inset). The alkaline pK_a's of dihydroxy BO-IMI and **6'** were found to be 10.0 and 10.8, respectively, by monitoring their electrophoretic mobility relative to that of BODIPY hydrazide (neutral marker of electroendosmosis) as a function of pH (data not shown).

In Figure 6 is shown the separation by CE of BO-IMI conjugates of normal deoxynucleotides, two DNA adducts (compounds **8** and **9**) and also a synthetic precursor, **7**, for the preparation of **9**. The inset in this figure shows the electropherogram of a BO-IMI labeled DNA oligomer. This latter result was reported before (12), but the electropherogram was not shown. Each conjugate gives a single peak since the N1 and N3 isomers for each one co-migrate at alkaline pH. We have achieved the detection of 60 femtomoles of 5'-dAMP as shown by the electropherogram in Figure 7B. Corresponding electropherograms for the detection of 600 femtomoles of 5'-dAMP, and for a blank reaction, are shown in Figure 7A and 7C, respectively.

Target substances bound on an IMAC column typically are eluted by a gradient to a lower pH, or by the addition of imidazole, ammonium acetate, or a chelating agent like EDTA. For our purposes, involving preparative or analytical purification, a pH gradient was most attractive. Based on the extensive literature concerning IMAC of histidine–containing substances, we started the separation at pH 7.0 in 0.1 M sodium phosphate buffer, and selected to first test a Ni(II) form of IMAC.

Neither BO–IMI nor BO–HZ migrated on the Sepharose–IDA at pH 7.0 (visual observation of the yellow–green band of sample on the top of the column bed, which can be enhanced by exposure to a UV lamp, providing 365 nm), so we began a stepwise pH gradient employing 0.1 M sodium acetate buffer (HCl treated). The separation was monitored more quantitatively by collecting fractions and testing them off–line by CE–LIF (data not shown). These conditions nicely resolve the two compounds by Ni(II)-IMAC, with BO–IMI eluting much earlier than BO–HZ, as shown in Fig. 8A.

Similar testing of Cu(II) and Zn(II) forms of Sepharose–IDA gave the chromatograms displayed in Fig. 8B and 8C, respectively. As seen, the two compounds are separated under both of these conditions as well, but with a change in the order of retention on the Zn(II) column. We did not optimize any of the separations, since they were all adequate for our purposes. These chromatograms show that the decreasing order of retention is Cu(II) > Ni(II) > Zn(II) for BO–IMI, and for BO–HZ it is Cu(II) ~ Ni(II) > Zn(II).

We wish to speculate, in part, on the mechanisms producing the separations shown in Fig. 8. All three metal ions are medium–soft in their polarizability, so this concept does not seem to be helpful. It is known that hydrazides tend to bind as bidentates to metal ions, whereas imidazole acts as a monodentate ligand, so this could explain the stronger retention of BO–HZ than BO–IMI on the Ni(II) and Cu(II) packings. This speculation is represented in Fig. 9.

RECOMMENDATIONS

The next step in the development of our new methodology is to bring together our optimized labeling conditions and IMAC techniques so that our methodology can be applied to trace detection of DNA adducts in the proposed biological samples.

CONCLUSIONS

We have characterized in more detail our prototype fluorescent tag, BO-IMI, to help us optimize the use of this key reagent in our method. This has led to our detection of 60 fmol of dAMP. Potentially higher sensitivity can be achieved by taking advantage of immobilized metal affinity chromatography, now that we have studied BO-IMI in this respect as well.

Achieving high sensitivity is essential in the detection of DNA adducts, making this work on optimization important for our project.

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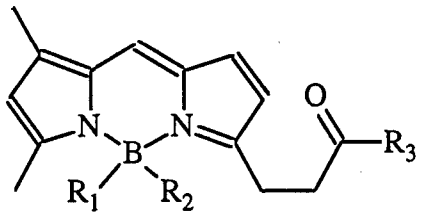
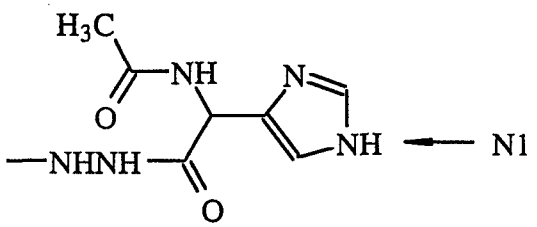
Table I Structures of BODIPY Derivatives



1 = BO-IMI

	R_1	R_2	R_3
1, 1'	F, OH	F, OH	X
2, 2'	F, OH	F, OH	X-5'-dAMP[a]
3, 3'	F, OH	F, OH	X-5'-dAMP[a]
4, 4'	F, OH	F, OH	$NHNH_2$
5, 5'	F, OH	F, OH	see Fig. 5
6'	OH	OH	$NHNHCOCH_3$[b]
7'	OH	OH	X-C8-benzyloxy-5'-dAMP[c]
8'	OH	OH	X-C8-AAF-5'-dGMP[c]
9'	OH	OH	X-C8-oxo-5'-dAMP[c]
10'	OH	OH	OH
11'	OH	OH	X-pCAAGCTTG

X =



[a]Phosphoimidazolides where one is N1 and one is N3 with respect to the imidazole moiety. Which isomer is the major product is unknown, however.

[b]Postulated structure.

[c]Mixture of N1 and N3 phosphoimidazolides with respect to the imidazole moiety.

FIGURE CAPTIONS

Fig. 1 Electropherograms at pH 6.0 (buffer B) showing two BO-IMI-5'-dAMP isomers (**2** and **3**) as a function of reaction time, which was either 0.3 (A) or 3.0 hours (B). Peak **1** is BO-IMI. Sample preparation: BO-IMI (2.4×10^{-2} M), dAMP (2.4×10^{-2}M), and EDC (1.8×10^{-1} M) in buffer A, 10 µl each, were combined.

Fig. 2 Effect of pH on BO-IMI-5'-dAMP. (A) Fresh reaction mixture of BO-IMI, dAMP and EDC. (B) Sample after incubation in Buffer D (pH 10.4) for 0.5 hour at room temperature. (C) Co-injection of A and B. Peaks: **1** = BO-IMI; **2** and **3** = BO-IMI-5'-dAMP; **4** = BODIPY FL C_3 hydrazide. **1', 2', 3', 4'** are the corresponding dihydroxy compounds. The separation was done at pH 6.0 (buffer B).

Fig. 3 Electropherograms of BO-IMI-5'-dAMP at pH 10.4 (buffer D). The sample (pH 6.0) was injected immediately (A) or 0.5 hour after its pH was adjusted to pH 10.4 by dilution (1:100) into buffer D (B). Peak identification: see Table 1.

Fig. 4 Electropherograms of BO-IMI-EDC, **5'** (structure in Figure 5) at pH 10.4 (buffer D). The sample (a reaction mixture at pH 6.0) was either: A, diluted into buffer D and injected 30 min later; or B, kept first in 0.1 M HCl for 5 h prior to this procedure. Inset: electropherogram at pH 10.4 (buffer D) of dihydroxy-BODIPY FL C_3 hydrazide, 4', and an N-acetylated derivative, 6'. Structures: see Table 1.

Fig. 5 Mass spectrum (capillary liquid chromatography continuous-flow fast atom bombardment) of BO-IMI-EDC, **5**. The solvent was 1% glycerol in 0.01 M ammonium acetate.

Fig. 6 Electropherogram at pH 10.4 (buffer D) of dihydroxy-BO-IMI conjugates of 5'-dAMP (**A**), 5'-dCMP (**C**), TMP (**T**), 5'-dGMP (**G**), C8-benzyloxy-5'-dAMP (**7'**), C8-[N-acetyl-N-(2-fluorenyl)]amino-5'-dGMP (**8'**), and C8-hydroxy-5'-dAMP (**9'**). Peak **1'** is dihydroxy-BO-IMI. Inset, corresponding dihydroxy BO-IMI conjugate of the oligodeoxynucleotide **5'**-pCAAGCTTG (**11'**), at pH 8.7 (buffer C, which was in use at the outset of our project). Sample preparation: BO-IMI (1.2×10^{-2} M), total adducts (1.2×10^{-2} M), and EDC (9×10^{-2} M), all in buffer A, 10 µl each, were mixed, and the resulting solution was kept unstirred in the dark at room temperature for 2 h followed by filtration through

a Pasteur pipet column containing ~140 mg propyl sulfonic acid silica retained on glass wool.

Fig. 7 Electropherograms obtained by reacting 600 (A), 60 (B) and 0 (C) femtomoles of 5'-dAMP with BO-IMI followed by cation exchange filtration, evaporation, addition of buffer D, incubation for 30 min, and injection. Peak assignments: see Table I.

Fig. 8. IMAC chromatograms obtained by loading a Sepharose-IDA column in the Ni, Cu, or Zu form, as indicated, with a solution of BO–IMI and BO–HZ at pH 7.0, and eluting with stepwise changes to lower pH while monitoring the colored bands visually, and also collecting 2 ml fractions for measurement of BO–IMI and BO–HZ by CE–LIF.

Fig. 9. Postulated bidendate binding of BO–HZ to the Ni(II) and Cu(II) forms of Sepharose IDA. L = arbitrary ligand

Figure 1

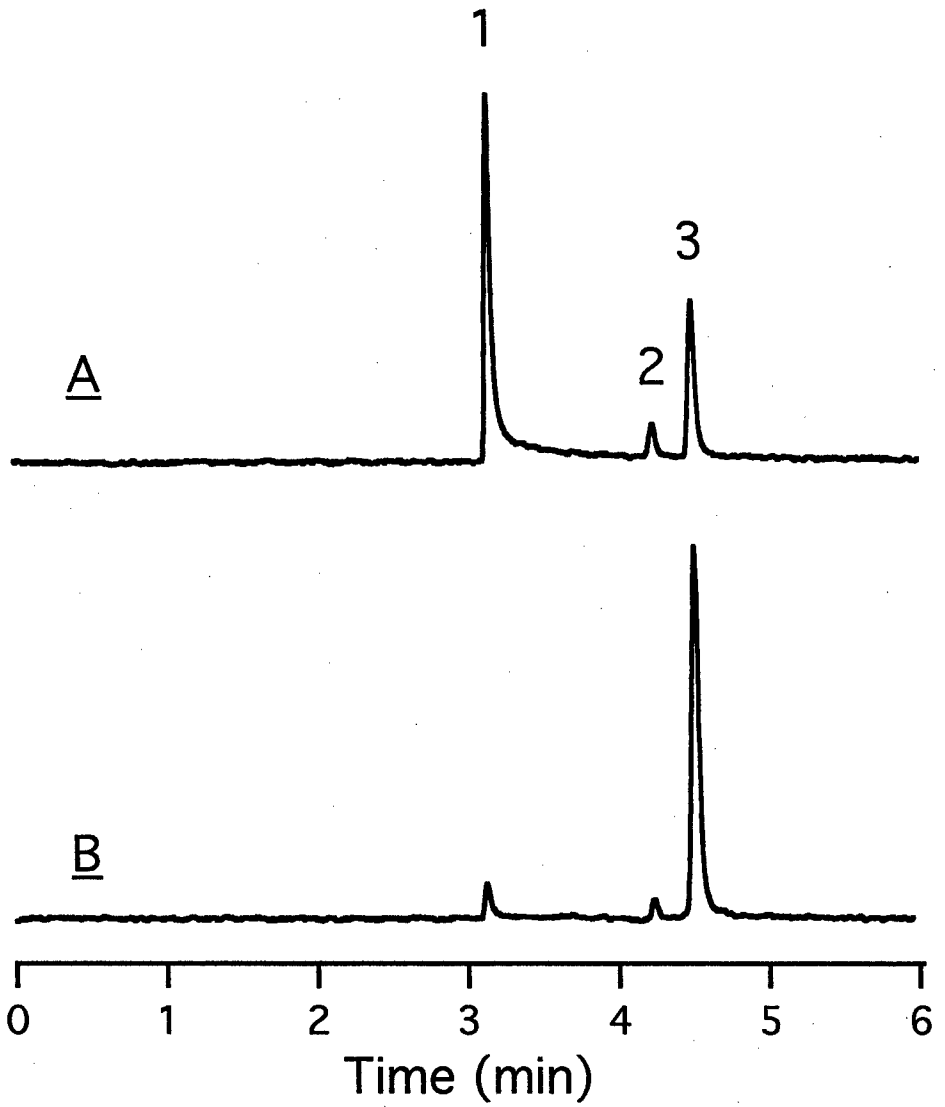


Figure 2

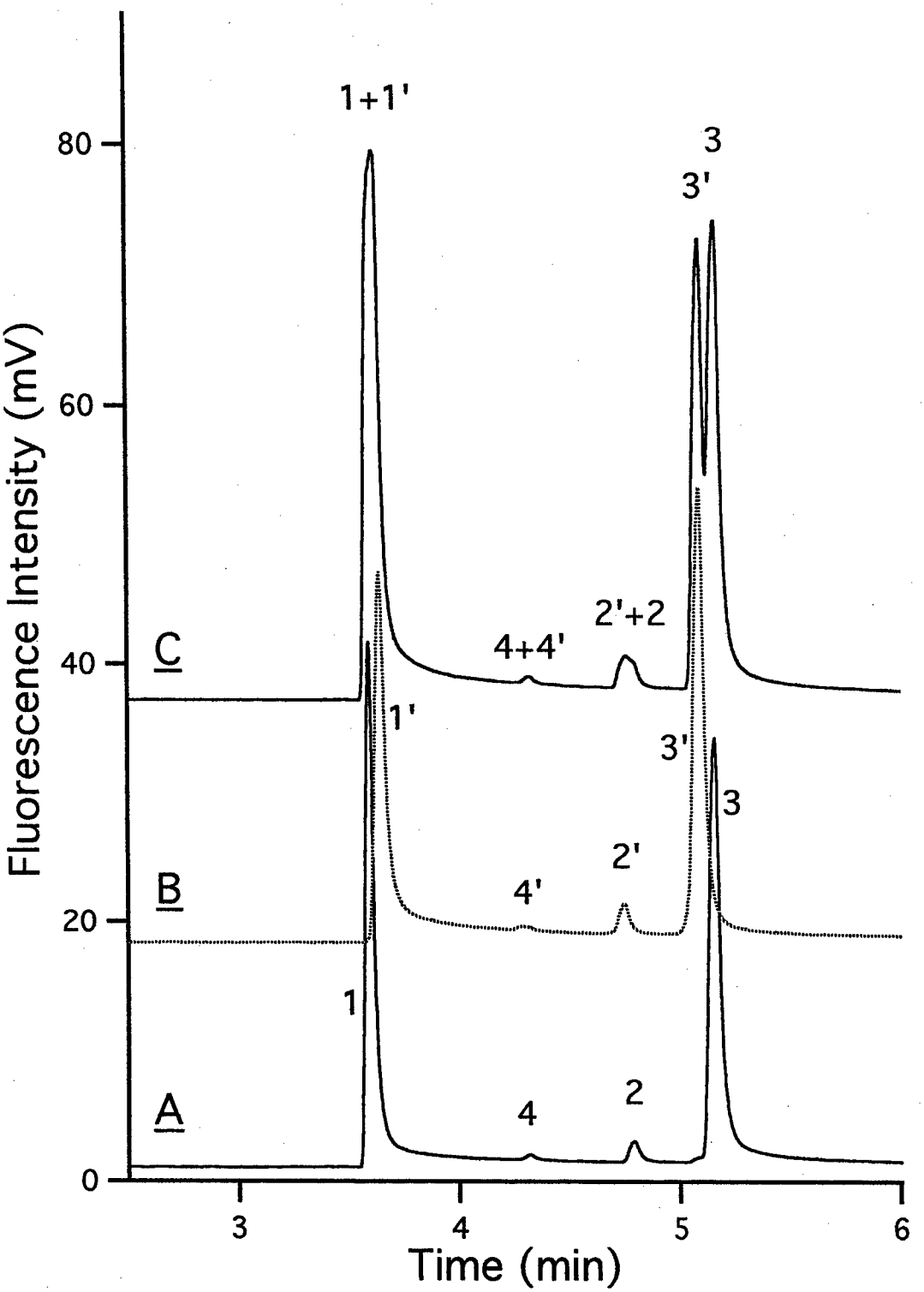
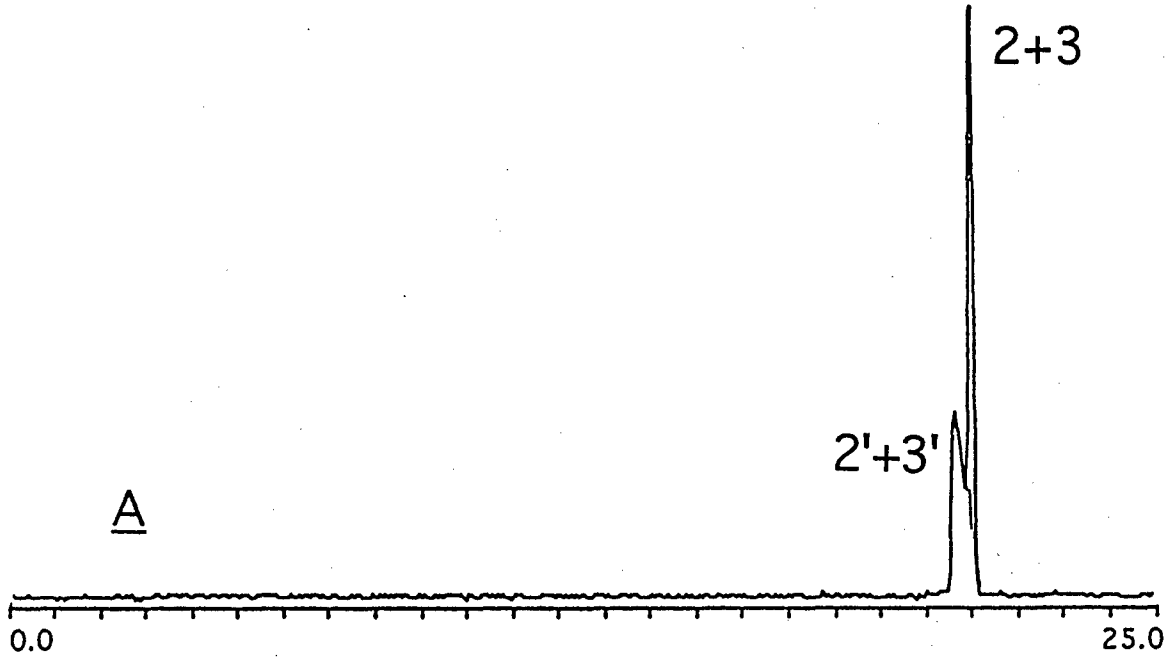

Fluorescence Intensity (mV)
80
60
40
20
0
1+1'
3
3'
2'+2
4+4'
C
1'
3'
3
2'
4'
B
1
A
4
2
3
4
5
6
Time (min)

Figure 3



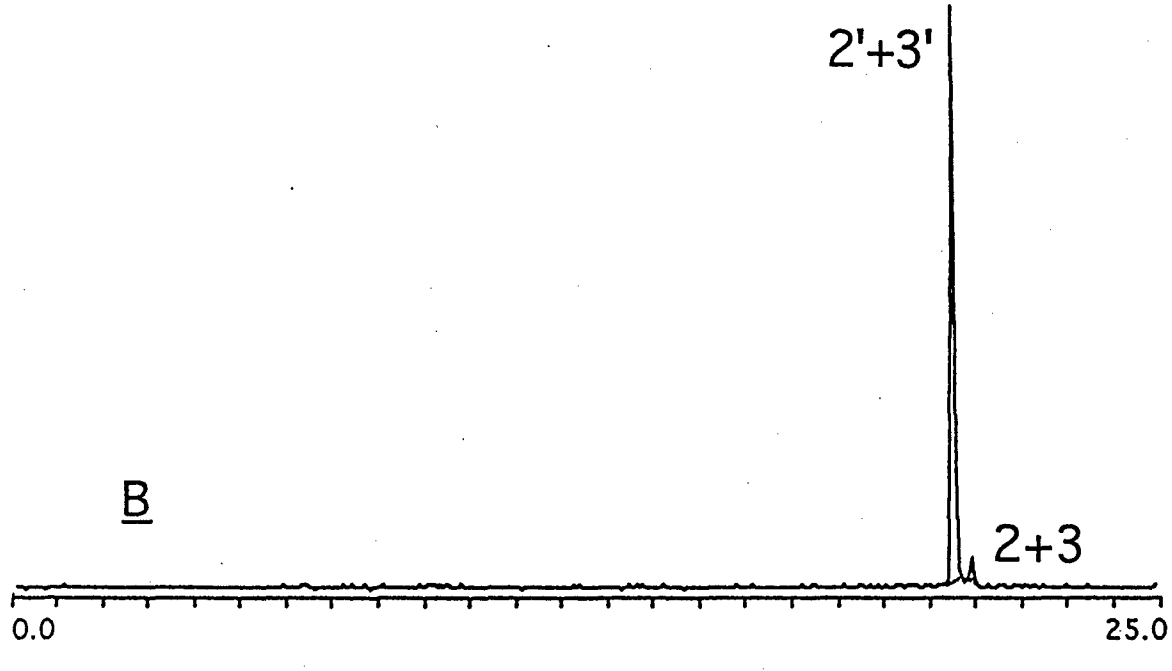


Figure 4

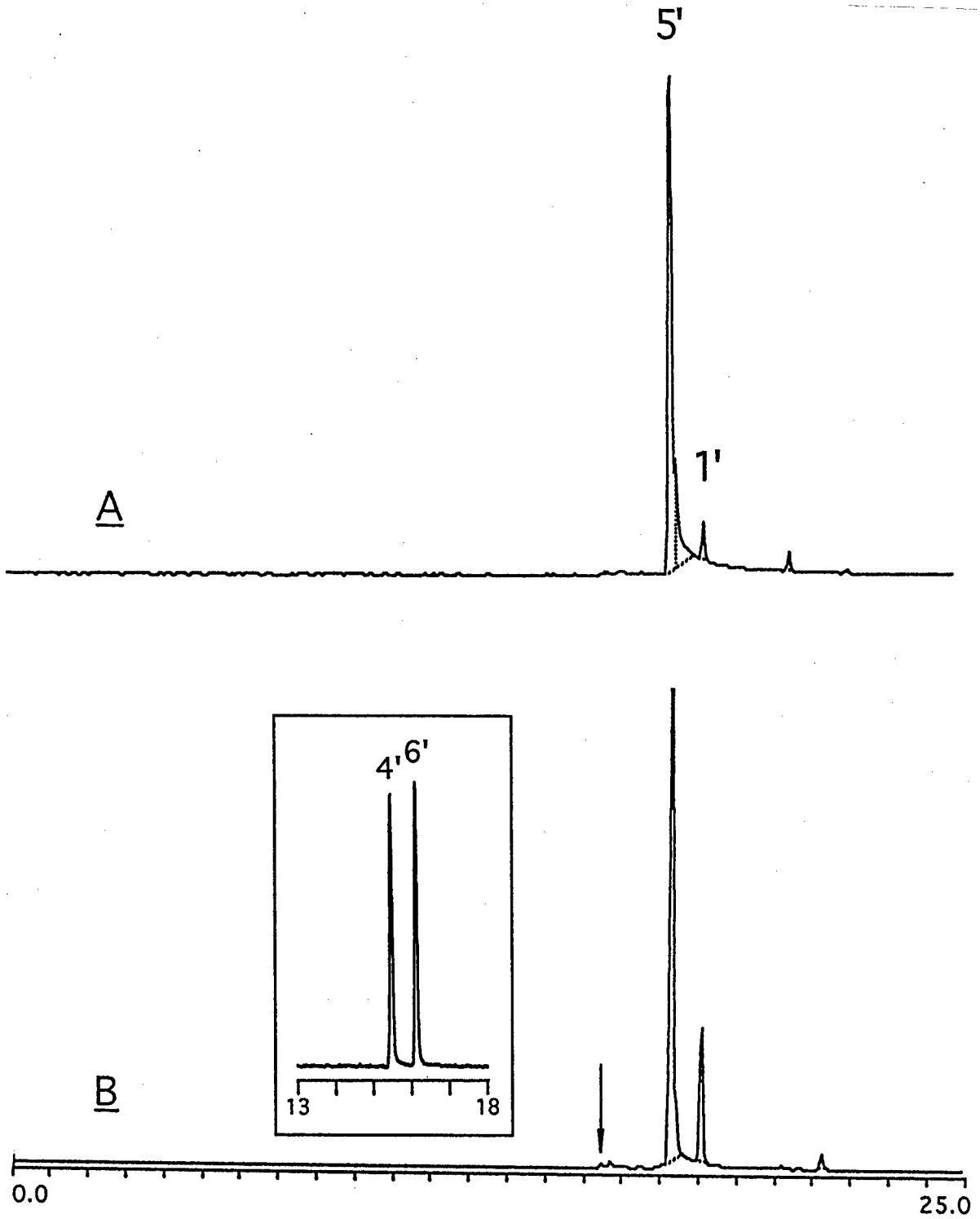


Figure 5

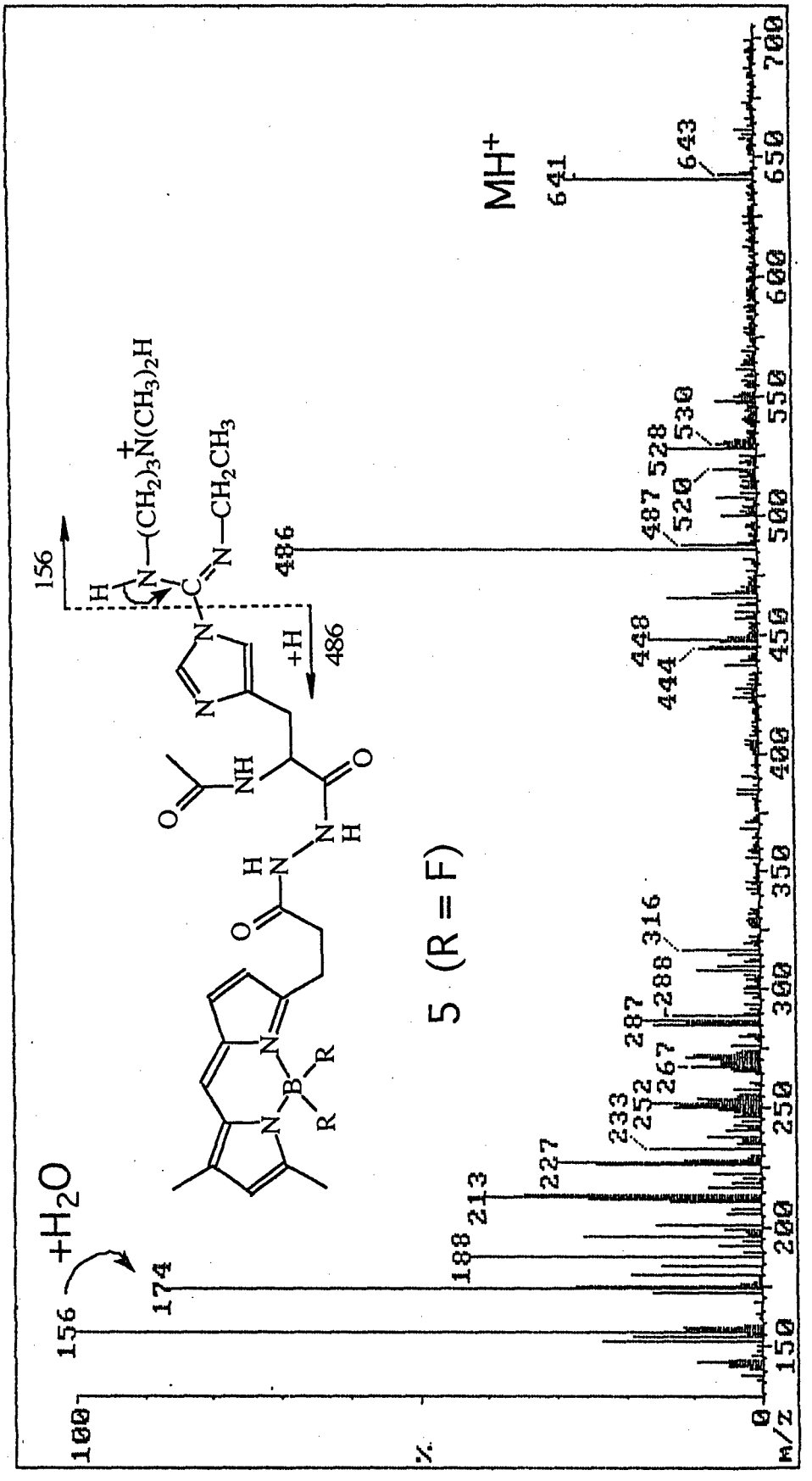

100
%
0
m/z
150
200
250
300
350
400
450
500
550
600
650
700
156
+H2O
174
188
213
227
233
252
267
287
288
316
444
448
486
487
520
528
530
641
643
MH+
5 (R = F)
+H
156
486

Figure 6

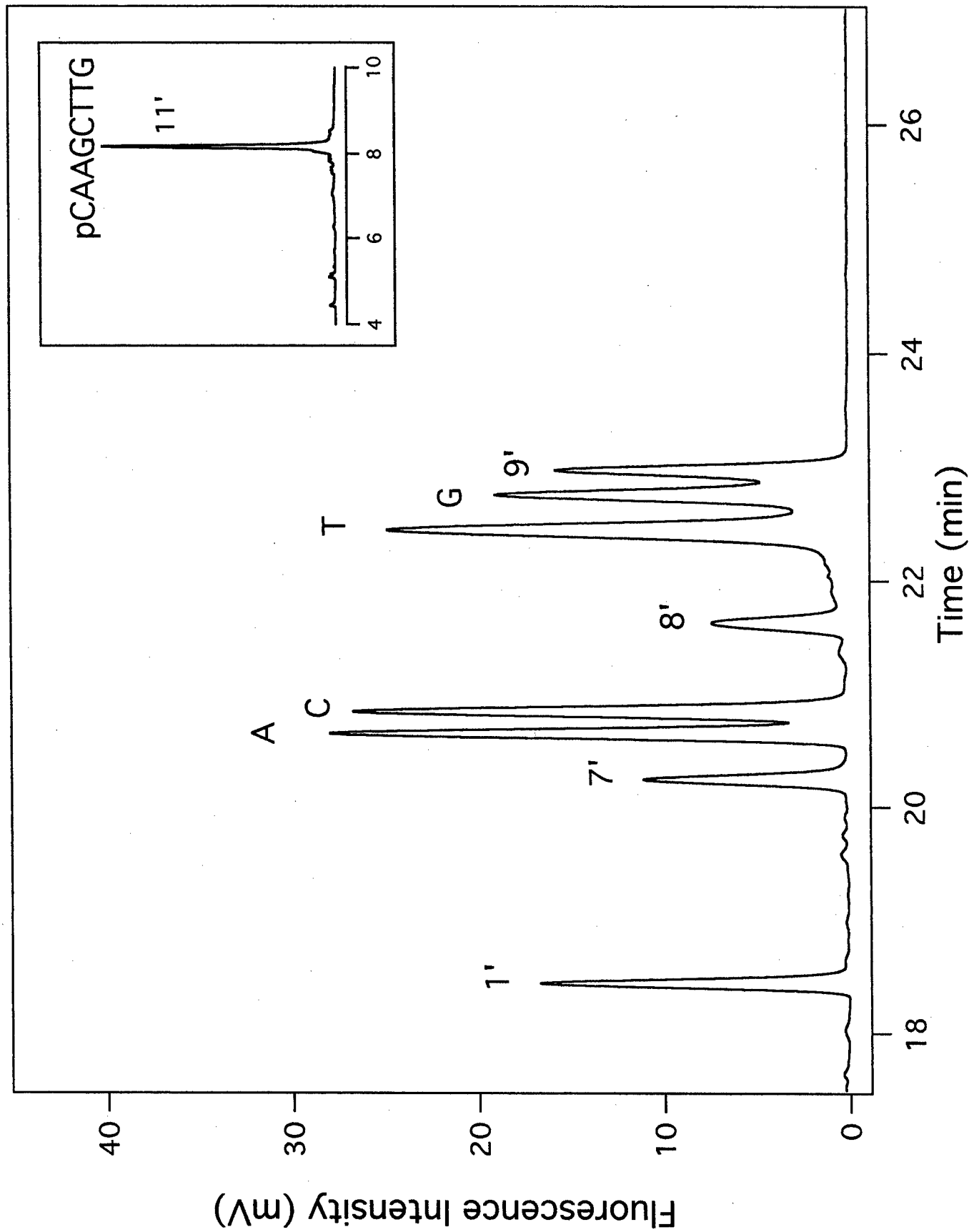

Fluorescence Intensity (mV)
0
10
20
30
40
18
20
22
24
26
Time (min)
1'
A
C
7'
T
G
8'
9'
pCAAGCTTG
11'
4
6
8
10

Figure 7

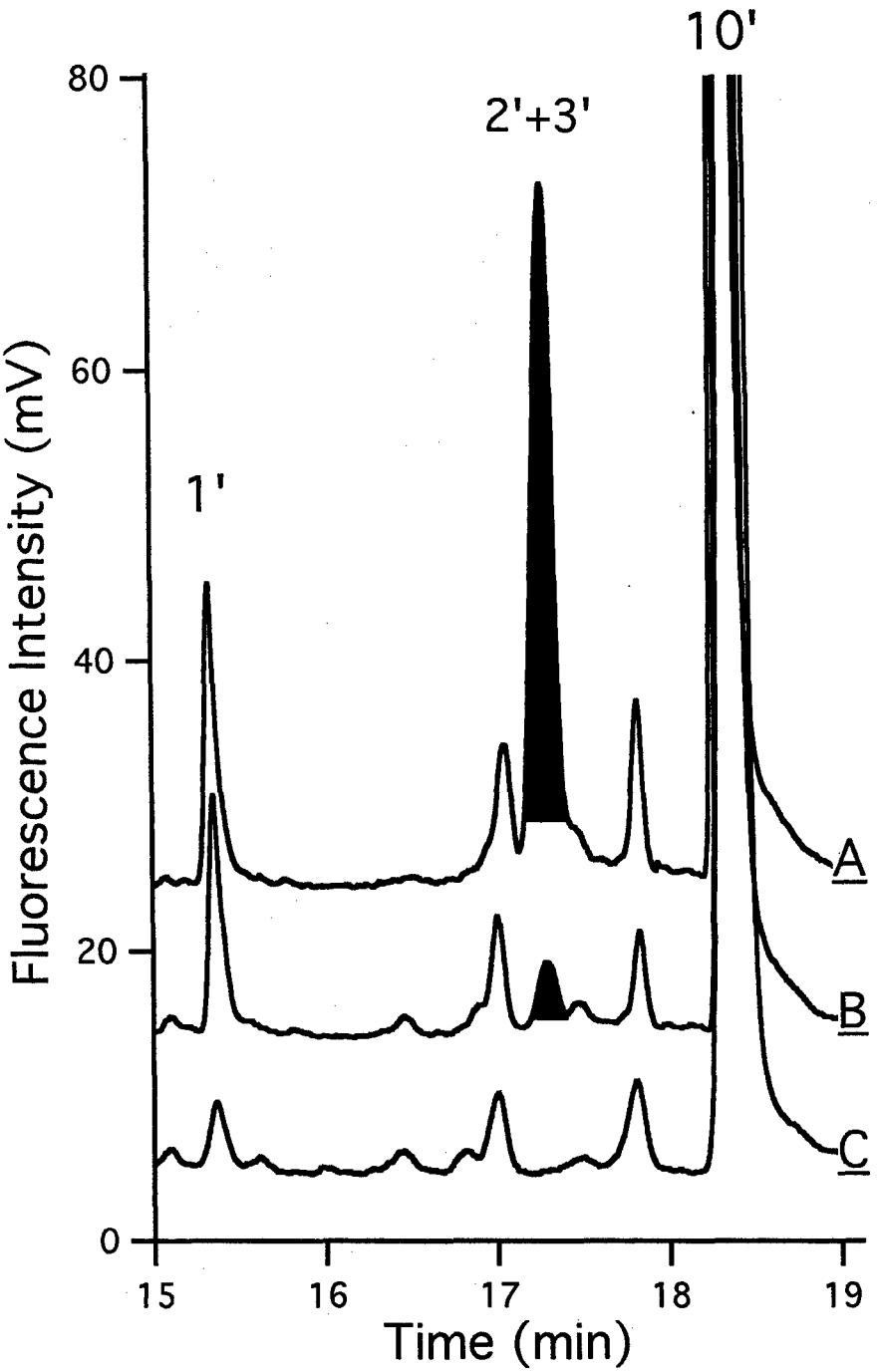

10'
2'+3'
1'
80
60
40
20
0
Fluorescence Intensity (mV)
A
B
C
15
16
17
18
19
Time (min)

Figure 8

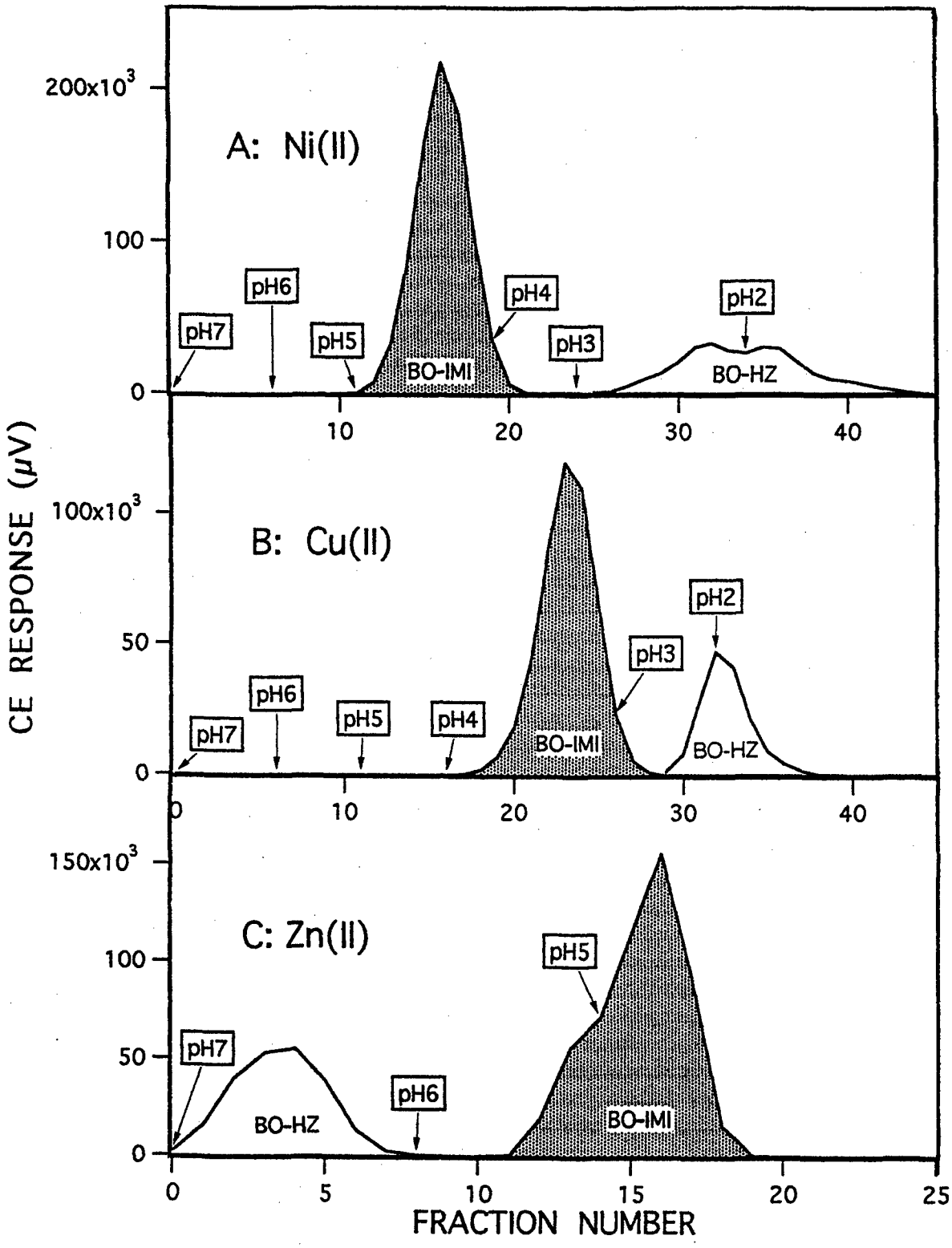
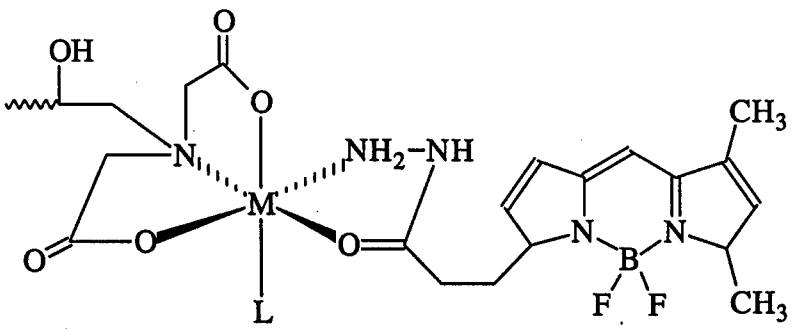

A: Ni(II)
B: Cu(II)
C: Zn(II)
pH7
pH6
pH5
pH4
pH3
pH2
BO-IMI
BO-HZ
200x10³
100
0
100x10³
50
150x10³
10
20
30
40
5
25
CE RESPONSE (μV)
FRACTION NUMBER

Figure 9



M = Cu (II); Ni (II)

L = arbitrary ligand

Rec'd
7/19/2000

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US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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DAMD17-94-J-4057	ADB221437, ADB247857
DAMD17-96-1-6069	ADB230256, ADB239319
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